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Decreased ethyl carbamate generation during Chinese rice wine fermentation by disruption of *CAR1* in an industrial yeast strain

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ABSTRACT

Saccharomyces cerevisiae metabolizes arginine to ornithine and urea during wine fermentations. In the fermentation of Chinese rice wine, yeast strains of *S. cerevisiae* do not fully metabolize urea, which will be secreted into the spirits and spontaneously reacts with ethanol to form ethyl carbamate, a potential carcinogenic agent for humans. To block the pathway of urea production, we genetically engineered two haploid strains to reduce the arginase (encoded by *CAR1*) activity, which were isolated from a diploid industrial Chinese rice wine strain. Finally the engineered haploids with opposite mating type were mated back to diploid strains, obtaining a heterozygous deletion strain (*CAR1*/*car1*) and a homozygous defect strain (*car1*/*car1*). These strains were compared to the parental industrial yeast strain in Chinese rice wine fermentations and spirit production. The strain with the homozygous *CAR1* deletion showed significant reductions of urea and EC in the final spirits in comparison to the parental strain, with the concentration reductions by 86.9% and 50.5% respectively. In addition, EC accumulation was in a much lower tempo during rice wine storage. Moreover, the growth behavior and fermentation characteristics of the engineered diploid strain were similar to the parental strain.

1. Introduction

Ethyl carbamate (EC), known as a potential carcinogenic compound, is widely found in alcoholic beverages in significant amounts (Ough, 1976; Ough et al., 1988; Wu et al., 2012). Chinese rice wine typically fermented by the rice is a popular traditional alcoholic beverage with a long history in China. A survey of EC in Chinese rice wine from Zhejiang province in China showed that rice wine contained EC at a range of 8–515 μg/kg with an average of 160 μg/kg (Wu et al., 2012). Due to its toxicity, carcinogenicity, and universality, EC is currently one of the biggest challenges in the alcoholic beverages industry. A large amount of research has been carried out on the mechanism of EC formation and strategies of preventing EC accumulation in alcohol beverages over the past few decades.

Based on previous research, there are at least five precursors that can be converted into EC, including urea, citrulline, carbamyl phosphate, cyanogen and diethyl pyrocarbonate in different kinds of alcoholic beverages (Ough et al., 1988; Wu et al., 2012). Among them, urea is the most important EC precursor in most alcoholic beverages, such as Chinese rice wine, sake and grape wine (Ough, 1976; Weber and Sharypov, 2008). Urea is mainly accumulated in yeast *Saccharomyces cerevisiae* by arginase-dependent degradation pathway. Urea can be exported into the surrounding medium where it reacts with ethanol to form EC with a moderate kinetic formation spontaneously during alcoholic beverages' fermentation and storage (Monteiro et al., 1989; Ough et al., 1990; Weber and Sharypov, 2008).

Current available methods to reduce EC concentration in alcoholic beverages can be classified into physical, chemical, enzymatic, and metabolic engineering routes as summarized by Zhao et al. (2013). And it has been recognized that metabolic engineering technologies provide novel methods for repressing the production of urea or enhancing the metabolic capability of urea in yeast, and thus that of EC. Arginase encoded by the *CAR1* gene in *S. cerevisiae* has become the first target of metabolic modification to reduce urea production, and hence the EC concentration. Recent works have constructed *car1* null mutant yeasts to reduce EC concentration in two alcohol beverages, sake and cherry spirits. A diploid yeast strain (*car1*/*car1*) for sake production was constructed using two dominant resistance cassettes and was shown to completely eliminate urea and EC in sake fermentations, even after storage for 5 months at 30 °C (Kitamoto et al., 1991). Another diploid

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Table 1
S. cerevisiae strains used in this study.

Strain	Parental	Genotype	Source
N85		*MATa/MATα*	NERCCRW[a]
Na	N85	*MATa*	Wu et al. (2013)
Nα	N85	*MATα*	Wu et al. (2013)
Na-u	Na	*MATa ura3*	This study
Nα-u	Nα	*MATα ura3*	This study
Na-c	Na-u	*MATa ura3 car1::URA3*	This study
Nα-c	Nα-u	*MATα ura3 car1::URA3*	This study
N85-c1	Na/Nα-c	*MATa/MATα URA3/ura3 CAR1/car1::URA3*	This study
N85-c2	Na-c/Nα-c	*MATa/MATα ura3/ura3 car1::URA3/car1::URA3*	This study

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laboratory yeast strain, which was shown to be capable of significantly reducing EC in cherry spirits by approximate 60%, was obtained with the same approach (Schehl et al., 2007). However, the constructed *car1* null mutants either introduced exogenous genes even antibiotic genes that would cause food safety problems, or were laboratory strains with low fermentation characteristics that restricted their application in food industries.

In our previous work, haploid strains of the industrial yeast *S. cerevisiae* N85 used in Chinese rice wine brewing have been isolated for further metabolic engineering to improve its fermentation performance and reduce harmful by-products (Wu et al., 2013). In the present study, strains with either a heterozygous deletion (*CAR1*/*car1*) or a homozygous defect (*car1*/*car1*) were constructed from the started haploid strain with a *CAR1* disruption cassette, using *URA3* as a selection marker. Therefore, the engineered strain was fit to commercial use since there were no exogenous genes or antibiotic genes introduced during genetic manipulation. The capability of suppressing EC accumulation of the engineered strains was validated. Moreover, the arginase activity, growth behavior and fermentation characteristics of the engineered diploid strains were compared with the parental strain.

2. Materials and methods

2.1. Strains and medium

Escherichia coli strain JM109 stored in our laboratory used in all cases for vector propagation was cultivated aerobically at 37 °C in Luria–Bertani medium (LB), 2% (w/v) agar and 50 μg/ml ampicillin (Sangon, Shanghai, China) were added when requiring. All the *S. cerevisiae* strains constructed in the present study were described in Table 1. The parental strain N85 is a commonly used industrial diploid yeast strain in Chinese rice wine brewing stored in National Engineering Research Center for Chinese Rice Wine. The haploid strains were obtained in our previous work (Wu et al., 2013). Yeast strains were grown at 30 °C in yeast peptone dextrose medium (YPD) which was solidified with 2% (w/v) agar when necessary. Minimum medium (MM) (0.17% (w/v) YNB (Yeast Nitrogen Base without Amino Acids), 2% (w/v) glucose, 0.5% (w/v) ammonium sulfate, 2% (w/v) agar) was used for culturing prototrophic strains. 0.005% (w/v) uracil was supplemented to the MM as SM-Ura medium for the culturing of Δ*ura3* mutants. And 5-FOA medium, SM-Ura supplemented with 0.125% (w/v) 5-FOA (5-fluororotic acid) was used for the selection of Δ*ura3* mutants. Sporulation was performed on potassium acetate (KAc) medium (1% (w/v) KAc, 2% (w/v) agar) for 3 days at 26 °C to verify the diploid strains.

2.2. DNA manipulation, disruption cassette and strain construction

Primers used in the present study were all designed according to the sequence of *S. cerevisiae* S288c genome (*SGD*, http://www.yeastgenome.org/) and listed in Table 2. DNA templates used for PCR amplification of yeast genomic sequences were isolated from strain N85. Plasmid DNA extraction was accomplished using commercial DNA purification kits (Takara, Dalian, China). Plasmid constructs and PCR products were verified by DNA sequencing (Sangon, Shanghai, China).

To use the *URA3* gene as a selection marker in the following *CAR1* disruption experiment, auxotrophic uracil haploid strain (Δ*ura3*) was constructed. The *URA3* disruption cassette was constructed by fusion PCR according to one-step gene disruption method (Rothstein, 1982). The upstream (*URA3*-U) and downstream (*URA3*-D) fragment of *URA3* was amplified from N85 genomic DNA using primer pair P1/P2 for *URA3*-U and P3/P4 for *URA3*-D, respectively. Subsequently, *URA3*-U and *URA3*-D were purified with Mini BEST Agarose Gel DNA Extraction kit (Takara, Dalian, China) and pooled with equimolar amount as template for fusion PCR using primer pair P1/P4 to construct the *URA3* disruption cassette, which was then purified and sequenced.

The *URA3* disruption cassette was electro-transformed into haploid strains Na and Nα according to the high-efficiency protocol (Gietz and Schiestl, 1991; Wu and Letchworth, 2004). Transformants were incubated for 2 h at 30 °C 100 r/m before plating on the 5-FOA plate, and then were replicate plated on MM plate. Colonies that could grow on 5-FOA medium but not on the MM medium were selected for colony PCR identification as auxotrophic uracil strain (Δ*ura3*) with primer pair P1/P4 and further verified using growth test for at least three times.

The *CAR1* disruption cassette was constructed as follows. First of all, the *CAR1* gene was amplified with primer pair P7/P8 and then cloned into plasmid pMD18-T (Takara Dalian, China) to generate plasmid pMD18-*CAR1*. Secondly, *URA3* gene was amplified with primer pair

Table 2
Sequences of the primers used in this study.

NO.	Name	Sequences (5′ → 3′)	Product length (bp)
P1	URA3-1_p	GTAATCTCCGAGCAGAAG	
P2	URA3-1_t	GGTGTCATAATCAACCAATCATTCGTAATGTCTGCCCAT[a]	510
P3	URA3-2_p	ATGGGCAGACATTACGAATGATTGGTTGATTATGACACC[a]	416
P4	URA3-2_t	ATACTGTTACTTGGTTCTGGC	
P1	URA3-1_p	GTAATCTCCGAGCAGAAG	927 (Δ*ura3*)
P4	URA3-2_t	ATACTGTTACTTGGTTCTGGC	1150 (*URA3*)
P5	U-1	TCC**CCGCGG**GTTTCAGGGTCCATAAAGCT[b]	1253
P6	U-2	GGA**AGATCT**TGTTACTTGGTTCTGGCGAGGT[c]	
P7	C-1	CAGGACCTCATTACAACTAC	1201 (*CAR1*)
P8	C-2	CAAATTGAAGCAGGTGTAGATG	2195 (Δ*car1*)
P9	MAT	AGTCACATCAAGATCGTTTATGG	544
P10	MAT-a	ACTCCACTTCAAGTAAGAGTTTG	
P9	MAT	AGTCACATCAAGATCGTTTATGG	404
P11	MAT-α	GCACGGAATATGGGACTACTTCG	

[a] Overlapping sequences required for the fusion PCR are underlined.
[b] *Sac* II sites indicated in bold.
[c] *Bgl* II sites indicated in bold.